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VOLUME V NUMBER 3

STAIN TECHNOLOGY

JULY, 1930

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Published by the

COMMISSION ON STANDARDIZATION OF BIOLOGICAL STAINS

Entered as second-class matter at the Post Office, Geneva, N. Y.

STAIN TECHNOLOGY

ISSUED QUARTERLY SUBSCRIPTION PRICE $1.50 PER YEAR

Editor and Business Manager
H. J. CONN
Soil Bacteriologist, N. Y. Agr. Exp. Station

Consulting Editors
CHEMISTRY: W. C. HOLMES Color Lab., U. S. Dept. of Agriculture
ZOOLOGY: S. I. KORNHAUSER School of Medicine, Univ. of Louisville
PATHOLOGY: F. B. MALLORY Boston City Hospital
BOTANY: W. R. TAYLOR Univ. of Michigan

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Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must ordinarily deal with the nature or uses of biological stains. Articles dealing with microscopic technic in general, however, will be accepted in most cases, if they have a close bearing on staining procedures. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

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Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which can be used as an author's abstract by abstracting journals. This summary must therefore be complete in itself, and must contain all the important facts brought out in the paper.

All illustrations should accompany the manuscript and should be numbered with the legend for each written on separate paper and attached loosely to the illustration. They should always be referred to in the text. Line drawings must be made in India ink. Photographs for half-tones must be on paper giving black and white tones and should preferably have a glossy finish.

Authors are especially urged to make all staining formulae definite and accurate. If known to the author, the dye content of the stain should be given and the exact dye used should be specified. If this information is not known, the name of the manufacturer should be stated and that of the stain itself exactly as worded on the label. Certified stains should be specified by certification number.

STAIN TECHNOLOGY

VOLUME V JULY, 1930 NUMBER 3

PROGRESS IN THE STANDARDIZATION OF STAINS

CANCER RESEARCH

At the Annual Meeting of the Executive Committee of the Commission on Standardization of Biological Stains, which was held in New York City on April 15th, one of the most interesting matters presented was an account of the cancer research which is being carried on under the direction of Dr. J. C. Bloodgood at Baltimore. The popular mind is so attracted today by certain methods for treating cancer or even by reputed cures, that not enough attention is paid to the actual progress that is being accomplished in improving methods of diagnosis, and in making earlier diagnosis possible. This progress is a real advance; and there is no question but that when a properly trained diagnostician is present during an operation the up-to-date surgeon today can obtain valuable evidence as to the presence and extent of malignancy that was not formerly available. Much more work, however, along this line is still necessary.

It will be recalled by many of our readers that something over a year ago an announcement appeared in *Science* and in the press of a very liberal grant of money made by Mr. Francis P. Garvan to Dr. J. C. Bloodgood to be used in the investigation of dyes in their relation to cancer. It is very well known now that perhaps the chief ambition of Mr. Garvan, President of the Chemical Foundation, is to promote the efforts of chemistry in the relief of human suffering. His activities in this connection have been numerous and varied. There is little doubt, in fact, but that this thought was one of the motives leading him to assure the Stain Commission the very generous backing it has been given by the Chemical Foundation; and it is certain that this was his chief object in the appropriation granted Dr. Bloodgood.

As a result of this appropriation the Garvan Cancer Research Laboratories have been established at the Johns Hopkins Hospital and Medical School. These laboratories are considering various phases of the possible relation between dyes and cancer. Perhaps it is too much to hope that dyes will prove of value in the treatment

of this disease or that a dye can be found which is actually specific for the cancer cell and which, therefore, will make the microscopic diagnosis absolute. Neither of these results is impossible, however, and we can certainly anticipate great improvement in staining methods, which will make diagnosis more certain than at present. Dr. C. F. Geschickter, who is in charge of this work, was present at the meeting of our Executive Committee, chiefly to report what has already been accomplished in this line. Some of the results, which have led to an improved technic for staining fresh tissue are reported elsewhere in this number of STAIN TECHNOLOGY. The method described, together with certain others that are not yet quite ready for publication, proved very interesting to the members of the committee. Dr. Geschickter is positive, however, that the work is only at its beginning and that even more valuable procedures may be developed as it continues. He has promised to send reports of all such technical developments in the way of technic to STAIN TECHNOLOGY for publication.

A NEW MEMBER OF THE EXECUTIVE COMMITTEE

At the meeting of the Executive Committee on April 15th attention was called to the fact that the American Association of Anatomists has not been for some time actively represented on the Commission on Standardization of Biological Stains. It is entitled to such representation and as the annual meeting of this Society occurred only two days after our Executive Committee meeting, a special invitation was extended to that organization to appoint a representative. The Anatomists acted on the suggestion and selected Dr. H. E. Jordan of the University of Virginia to represent them on the Commission.

The constitution of the Stain Commission specifies that the Executive Committee shall be composed of five to eight members, to be elected from such Society representatives. As there were only seven members on the Executive Committee at the time of our recent meeting, Dr. Jordan's name has now been proposed to the members of the committee as an eighth member thereof, and he has been duly elected to fill that vacancy. We accordingly extend our greetings to Dr. Jordan, as to one whom we are sure will prove a valuable member of the committee.

H. J. CONN

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the April number of this journal.

Stains Certified February 15 to May 15, 1930*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Eosin Y	LE 6	87%	As histological stain and as constituent of blood stains.	Feb. 26, 1930
Congo red	NQ 1	86%	As histological counter-stain	Mar. 1, 1930
Hematein	FHi 2	——	As histological and cytological stain	Mar. 24, 1930
Sudan III	NY 3	80%	As fat stain	Mar. 25, 1930
Basic fuchsin	CF 10	92%	As bacteriological and histological stain (not for use in Endo medium).	Apr. 1, 1930
Hematoxylin	FH 8	——	As histological and cytological stain	Apr. 17, 1930
Brilliant cresyl blue	NV 9	65%	As vital stain for blood	Apr. 24, 1930
Neutral red	CX 3	43%	As vital stain	Apr. 30, 1930
Methyl green	CG 4	48%	As histological stain	Apr. 30, 1930
Crystal violet	NC 9	92%	As histological and bacteriological stain	May 12, 1930
Gentian violet	CB 8	71%	As histological and bacteriological stain	May 12, 1930
Carmin	NCa 3	——	As histological and cytological stain	May 12, 1930
Fuchsin, basic	NF 17	94%	For staining and for use in the Endo medium	May 15, 1930

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

FRESH TISSUE DIAGNOSIS IN THE OPERATING ROOM

CHARLES F. GESCHICKTER

Garvan Cancer Research Laboratories, Johns Hopkins Hospital and University, Baltimore, Md.

ABSTRACT: **A polychromatic stain giving color reactions closely simulating hematoxylin and eosin, without overstaining and obscuring the finer detail of the cell, has been developed in this laboratory. It is as follows: To 4 parts of 1% aqueous azure A (azure I) which has been previously filtered, add very rapidly one part of filtered 0.5% aqueous Erie garnet B. The mixture is immediately filtered to prevent precipitation. Occasional refiltering may be necessary if the mixture has been standing a month or more. Float tissue slices from a freezing microtome directly into distilled water; pick them up with a round glass rod and immerse into the stain which has been poured into a small section dish. The staining time varies with different tissues but 10 to 15 seconds usually gives the most satisfactory results. Lift sections from the stain, still on the same glass rod, float thru two changes of distilled water, and then transfer to a clean glass slide. Place a large drop of 40% glucose on a clean cover slip and immediately invert over the section. The mounted specimen is then ready for microscopic examination. In examining the tissues under the microscope it is best to employ an intense artificial light (an ordinary 40 or 60 watt bulb is sufficient). After mounting, one-half to one minute should elapse before examining the section to allow it to clear.**

A valuable aid to the diagnosis of early cancer developed in recent years has been the method of freezing freshly excised tissue and cutting sections for microscopic examinations at the time of operation.[1] It has made possible increased accuracy of diagnosis in tumors by combining an examination of the microscopic appearances with the clinical and gross features of the lesion. But there is considerable discussion among a certain group of surgeons and pathologists, as to whether the patient is benefited in most instances by this earlier knowledge of the histology of the tumor on the part of the operator.

There is general agreement that fully developed cancer can be recognized clinically and in the gross, but it is likewise well recognized that this late type of case is most frequently hopeless. On the other hand, cases of early cancer which offer a definite percentage of curability, often show only microscopic evidence of malignancy.

[1]Bloodgood, J. C. Tissue diagnosis in the operating room. Southern Med. J., 21. 179–184. 1928.

Since tissue diagnosis in the operating room applies to this second type of case, its value depends upon the possibility of getting the patient with cancer to present himself to a competent physician in the earlier stages of the disease. Teaching the public the first warnings of malignant disease is essential to the accomplishment of this and the percentage of success derived is directly proportional to the educability of the lay-mind plus the degree and extent of symptomology manifested by various tumors in their earliest stage.

If the surgeon has the benefit of seeing patients in the early stage of a neoplastic disease, the next problem that arises concerns the curability of the various types of tumors in their incipiency. Surgeons are aware that the prognosis of certain neoplasms become increasingly worse as the duration of the symptoms become more brief and this is particularly true of certain types of sarcoma of the bone, where tumors with the shortest and most intense symptoms offer the worst prognosis.

Personal experiences with this sort of tumor have made certain clinicians extremely skeptical of the benefit gained by early diagnosis and intervention. This skepticism, however, is a matter of impression and not careful analysis. The confusion arises because of the inability of the diagnostician to grade or estimate the degree of malignancy of the tumor. Before and since the time of Broder's work, on the grading of epithelial tumors, the medical profession has become familiar in a general way with the fact that different neoplasms vary in their degree of malignancy from type to type, and also among the various tumors in the same type. Unfortunately, there is at present no body of information on which to base a reliable method of grading the degree of malignancy for every tumor. Nevertheless, clinicians should bear in mind that in judging the benefits to be derived from early intervention, it is only fair to compare tumors of equal degree of malignancy, and it is not at all rational to expect the same results from early intervention in a very high grade of malignancy that one would expect to obtain from one of relatively low degree of malignancy.

No matter how soon medical advice is sought, the competency of the physician consulted is a major factor in determining the benefits to be derived by the patient. Tumors should not be explored unless facilities for microscopic diagnosis and the complete operation for malignancy are available, if they should prove needed. When the physician consulted is sufficiently competent, and when the degree of malignancy is not too great, it gives the patient unquestionable advantage to be seen early by the physician. Only in cases of this

type can the true value of an immediate tissue diagnosis in the operating room be properly judged.

In such cases definite knowledge of the malignancy of the tumor at the time of operation permits immediate and radical procedures which would otherwise be delayed or substituted by insufficient treatment. On the other hand it prevents unnecessary mutilating operations for cancer when the condition is benign. The value of tissue diagnosis is greatest in border line cases where it is difficult to determine whether the condition is benign or malignant, and it is in such border line groups that much depends upon the adequacy of the technic of the tissue preparation and the pathologic skill of the one who interprets the microscopic findings. Improvements in the methods of sectioning and staining which would definitely increase the accuracy of diagnosis in this border line group of cases, would make the use of tissue diagnosis in the operating room more widespread.

Various methods of rapidly obtaining tissue from the operative specimen for microscopic examination have been advocated. Terry of the Mayo Clinic has advised free-hand sectioning with the razor or sharp scalpel. Dudgeon in London has advocated teasing the tissue on the slide. Perhaps the most valuable technic of all is the direct freezing and sectioning by the microtome without the employment of any fixative. By this method, sections from 10 to 15 microns in thickness can be obtained in from one to three minutes. Many cells are killed by this method, but preserve most of their finer structures and morphological detail nearly as well as tissue hardened in fixing solutions. The customary sections of fixed tissue, of course, cannot be employed because of the length of time required to make them, and the usual solutions of alcoholic dyes also cannot be used, because of the harm to the cells. The pathologist must, therefore, dispense with his familiar hematoxylin and eosin staining reaction and his celloidin and paraffin sections. Polychrome methylene blue has heretofore been resorted to as a substitute stain. This dye has the disadvantage, however, of giving an entirely different color reaction from that of hematoxylin-eosin, and also of overstaining so as to obscure the finer detail in the cell. If a stain were available which would closely approximate in its color combination that of hematoxylin-eosin and would at the same time not overstain, but depict the finer detail of the cell, accuracy in diagnosis would be much enhanced. Since speed is an essential requirement in the entire procedure, the stain would have to be a rapid one, accomplishing its results in a period of about fifteen seconds, and not over thirty.

A polychromatic stain having the above-mentioned properties has been developed in this laboratory. Staining is accomplished by immersing the frozen section in the dye for a period of from five to fifteen seconds, and the section is then mounted on a slide with aqueous glucose and the ordinary cover slip. Sections thus stained give better differentiation and more detail than the usual fixed tissue stained with hematoxylin-eosin; the stain is permanent and does not fade and will last as long as the section is preserved.

Pathologists competent to make a diagnosis on the usual hematoxylin and eosin section are in no way handicapped by the transfer to frozen section and this different stain. By the routine employment of such improved frozen section technic in the operating room, the surgeon has the benefit of an immediate reliable microscopic report, and can thus give the patient the advantage of the immediate radical operation, should the tumor prove malignant. In addition, when excising malignant neoplasms, repeated frozen sections can be made from various points of the operative field, to enable the surgeon to judge whether or not the disease is being completely removed, or has extended beyond the point of irradication.

Technic

The stain employed is a mixture of acid and basic dyes in approximately 1% aqueous solution. The basic dye used is azure A and the acid dye Erie garnet B. Both products were obtained from the National Aniline and Chemical Company of New York. These dyes must be mixed in certain proportions and by certain methods in order to prevent precipitation, since precipitation between such acid and basic dyes employed in the same mixture is the rule. The exact method of mixing these two dyes is as follows:

To 4 parts of a 1% aqueous solution of azure A which has been previously filtered, there is added one part of a filtered aqueous solution of 0.5% Erie garnet B. The Erie garnet B solution is added very rapidly and the resultant mixture is immediately filtered to prevent precipitation. It may be necessary from time to time to refilter the mixture if it has been standing a matter of weeks or months. This staining mixture is known in the Garvan Cancer Research Laboratory as "Metachrome No. 136".

It will be seen from the accompanying graphs, Fig. 1, that this mixture of azure A and Erie garnet B approximates in color effect hematoxylin and eosin. The absorption curve of Erie garnet B is maximal at 520 mμ, while that of the usual eosin Y mixture is maximal at 527. The absorption of hematoxylin in freshly prepared ammoniacal alcoholic solution is maximal at about 570, while that of azure A in aqueous solution is maximal at about 620.

The piece of tissue selected for microscopic examination preferably should not be much over a quarter of an inch in diameter (7 mm.) and about one eighth of an inch in thickness (3 to 4 mm.). If the piece of tissue is too large the size of the section cut will be difficult to handle without folding. The usual freezing microtome is employed, care being taken not to over-freeze the tissue which results in a hard and crumbly block that cuts poorly. (The preferable temperature is minus 10 to minus 1/10 degrees centigrade by actual

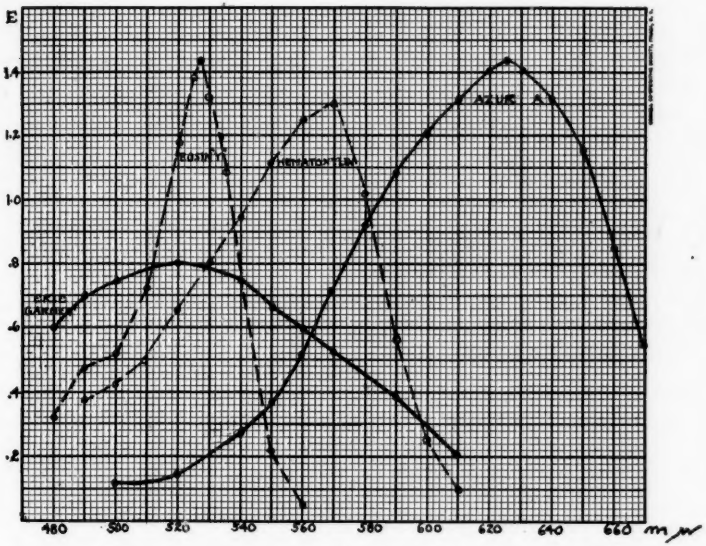

Figure 1. Absorption curves of Erie garnet B and azure A (azure I) in .005% and .001% respectively aqueous solution compared with eosin Y and hematoxylin (in freshly prepared ammoniacal) .005% alcoholic solution. The solution layers used were 0.5 cm. in thickness.

measurement.) The tissues are floated into distilled water as they come from the microtome and from there picked up by a round glass rod and immersed in the stain which has been poured into a small section dish. The staining time varies with different tissues but 10 to 15 seconds usually gives the most satisfactory results.

The sections are lifted from the stain, still on the same glass rod, and floated thru two changes of distilled water and then transferred to a clean glass slide. A large drop of 40% glucose is placed on a clean cover slip which is immediately inverted over the section and the

mounted specimen is then ready for microscopic examination. In examining the tissues under the microscope it is best to employ an intense artificial light (an ordinary 40 or 60 watt bulb is sufficient). After mounting ½ to 1 minute should elapse before examining the section to allow it to clear.

PRACTICAL METHODS FOR THE CONTROL OF HYDROGEN-ION CONCENTRATION IN STAINING PROCEDURES—THE USE OF "BUFFERS"

R. W. FRENCH

Army Medical Museum, Washington, D. C.

ABSTRACT: **Data are given for the simple preparation of dry chemical mixtures to be used in the easy preparation of buffer solutions for use in the control of stain procedures. Two of these mixtures of special value in practical staining operations are the following: 4.539 g. KH_2PO_4, 5.940 g. $Na_2HPO_4.2H_2O$. (pH=6.8); 7.262 g. KH_2PO_4, 2.376 g $Na_2HPO_4.2H_2O$ (pH=7.4). Hydrogen-ion control has been found to be essential to consistent results with the compound blood and tissue stains such as Wright's and Giemsa preparations. The more general use of a pre-determined pH in staining procedures is indicated; several possible methods of application are suggested.**

Suitable control of the hydrogen-ion concentration of many stain solutions may be accomplished by the use of simply prepared buffer mixtures. Buffer action is clearly defined by the following quotation from Clark:[1]

"If we were to add to 1 liter of perfectly pure water of pH 7.0, 1 cc. of 0.01N HCl, the resulting solution would be about pH 5.0 and very toxic to many bacteria. If, on the other hand, we were to add this same amount of acid to a liter of a standard beef infusion medium of pH 7.0, the resulting change of pH would be hardly appreciable. This power of certain solutions to resist change in reaction was commented on by Fernbach and Hubert (1900) who likened the resistance of phosphate solutions to a 'tampon.' The word was adopted by Sörensen (1908) and in the German rendition of his paper became 'Puffer' and thence the English 'buffer.' There has been some objection to this word as applied but it now possesses a clear technical meaning and is generally used. By buffer action we mean the resistance exhibited by a solution to change in pH thru the addition or loss of acid or alkali. . . ."

As customarily employed buffer solutions are made up to supply solutions of a known pH covering any range desired. These solutions are dilute and ordinarily require considerable space for storage. In staining procedures the use of so much bulk is often inconvenient.

[1]Clark, W. M. The Determination of Hydrogen Ions, 3rd Edition, Williams & Wilkins. Baltimore. 1928. See p. 50.

To overcome these difficulties and to make buffers of general use, a method has been devised of sufficient accuracy for the purpose by preparing mixtures of several salts in dry form by thoro mixing with a mortar and pestle. Then, suitable amounts of the mixture (approximately 50 mg. to 100 ml. of water, tap or distilled) is employed to secure pH stability.

Covering a range of pH 5.3 to 9.2 the phosphate mixtures of Sörensen[2] and the borax-boric acid mixtures of Palitzsch[3] are found practical, especially as the chemicals may be easily obtained of requisite purity and are easily handled. The following table gives the amounts of the several components required for a range of buffers in steps of 0.3 or 0.2 pH, while intermediate values may be interpolated as required:

pH	KH_2PO_4	$Na_2HPO_4+2H_2O$
5.3	0.227 grams	11.583 grams
5.6	0.454	11.286
5.9	0.908	10.692
6.2	1.816	9.504
6.5	2.723	8.316
6.8	4.539	5.940
7.0	5.447	4.752
7.2	6.355	3.564
7.4	7.262	2.376
7.7	8.170	1.188
8.0	8.624	0.594

	$Na_2B_4O_7+10H_2O$	H_3BO_3	NaCl
8.2	6.69 grams	8.06	1.89 grams
8.4	8.59	6.82	1.59
8.6	10.51	5.58	1.31
8.8	13.37	3.72	0.87
9.0	15.28	2.48	0.58
9.2	19.10	—	—

The potassium di-hydrogen phosphate, KH_2PO_4, borax, $Na_2B_4O_7+10H_2O$, boric acid, H_3BO_3, and sodium chloride, NaCl, as received are usually of sufficient purity for the purpose. The di-sodium hydrogen phosphate, is usually furnished with 12 molecules of water of crystallization and must be dried to the two molecule form,

[2]See Clark, Determination of Hydrogen Ions, 3rd Ed. 210.
[3]See Clark, Determination of Hydrogen Ions, 3rd Ed. 213.

$Na_2HPO_2+2H_2O$, before use. This is simply accomplished either by exposing the phosphate to ordinary room atmosphere for about two weeks, or by drying in the incubator at 37° C for two or three days. If this latter method is used, some care is necessary that the drying is not carried too far. The resulting phosphate should be a dry powder in contradistinction to the crystalline nature of the 12 molecule form.

For a given pH-value the several salts are mixed in the proportions indicated by the table. For instance, to prepare a mixture of pH 6.5, 2.723 grams of the potassium phosphate is mixed with 8.316 grams of the dried sodium phosphate in a mortar and about 50 mg. of the mixture added to about 100 ml. of water to make a buffered solution of pH 6.5. Ordinarily in the preparation of buffered solutions for stain procedures it is not necessary to weigh out the buffer salt mixture, as a little on the end of a knife blade will be sufficient to insure reasonable hydrogen-ion control in most instances.

Pre-determined hydrogen-ion concentration finds its application in the control of staining results with the Wright's, Giemsa or other compound stains of a similar nature. In general, staining in an acid solution will accentuate the action of the acid dyes, while staining in an alkaline bath will accentuate the basic dyes. In using the Giemsa mixture with tissue, for instance, it may be desirable to accentuate the methylene blue or azure components to bring out bacteria. This may be accomplished by preparing the Giemsa mixture for staining in a buffer solution at pH 7.4 or 7.7. On the other hand the connective tissue stain of eosin may be accentuated by staining at a pH of 6.2. A fair balance of the eosin-methylene-blue-azure components will be afforded by staining in a buffered solution of about pH 6.8.

Reliable performance of Wright's stain or the tetrachrome stain on blood films is assured by using a buffered solution of pH 6.5 or 6.8 for dilution of the alcoholic solution of the stain as well as in subsequent washing. This hydrogen-ion control will insure not only a good differential nuclear staining, but also a pink shade in the erythrocytes, which should be a characteristic of these stains when properly employed.

Some stain reactions may be influenced by a preliminary treatment of material with a buffer solution. Or, again, the stain may be mixed with the buffer to insure a pre-determined pH-value for staining; while in other procedures it may be found to be more desirable to employ the buffer in the differentiation, where hydrogen-ion concentration often plays an important part in obtaining consistent results.

It must be understood that when adding a mixture of buffer salts to a staining solution, or when using them either in pre-treatment or differentiation, one alters not only the hydrogen-ion concentration but also the salt content of the staining bath. This latter factor alone, regardless of changes in reaction, is known to have a decided influence in many cases on the behavior of dyes. The knowledge of this fact is not so necessary in the practical use of the above buffer mixtures as it is in the interpretation of the results obtained by their use. One should be cautious in ascribing such results wholly to alteration of hydrogen-ion concentration.

Control of acidity or alkalinity of alcoholic solutions is not always easily attained, tho buffers in water may be employed with satisfaction when diluting alcoholic solutions. Indications are that acidity or alkalinity of alcohols are of equal or greater importance, and in certain instances, especially with the Giemsa tissue stains, an absolute alcohol prepared with calcium carbide has been found superior to the usual product, no doubt because it is decidedly more alkaline than commercial samples of absolute alcohol.

THE ANALYSIS OF NEUTRAL RED AND OF THE PYRONINS

W. C. HOLMES[1] and A. R. PETERSON[2]

(Contribution No. 180 from the Color and Farm Waste Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington, D. C.)

ABSTRACT: **Neutral red and the pyronins cannot be evaluated by means of reduction with titanous chloride, and market supplies of the stains contain nitrogenous dye intermediates which render other chemical methods of determining dye content unreliable.**

Data are furnished in the present paper which will enable the analyst to obtain reliable dye-content values with samples of these dyes by means of a convenient spectrophotometric technic.

The volumetric method of reduction with titanous chloride is usually employed at this laboratory in the routine chemical analysis of biological stains. Whereas it affords a relatively convenient and reliable means for the evaluation of dyes in general, it does not appear to be applicable in the instances of neutral red, pyronin G and pyronin B. Since repeated attempts to devise a satisfactory technic for the quantitative reduction of these three important stains invariably failed, an investigation of other possible methods of analysis was undertaken.

Four methods were found of sufficient promise to warrant consideration.

THE KJELDAHL METHOD

Altho the great majority of dyes contain nitrogen, and altho none of the substances ordinarily mixed with dyes by manufacturers in their standardization are nitrogenous, the practicability of evaluating technical dye products on the basis of nitrogen determinations does not appear to have received previous investigation.

The Kjeldahl methods of the Association of Official Agricultural Chemists were employed, and the Kjeldahl-Gunning-Arnold technic adopted after determining that any preliminary reduction of the dyes under investigation was unnecessary. Excellent agreement in results was obtained, and it was established that the Kjeldahl method could be depended upon to give reliable nitrogen values with samples of all three dyes.

The evaluation of dyes on the basis of nitrogen content is direct in character, that is, the dye content (in the absence of interfering impurities) may be calculated directly from the analytical data. This is impossible with two of the other analytical methods subse-

[1]Senior Chemist, Color and Farm Waste Division, Bureau of Chemistry and Soils, Washington, D. C.

[2]Research Associate, Commission on Standardization of Biological Stains.

quently referred to, and is probably inadvisable in the third (the method of titration with naphthol green). The following methods, accordingly, were standardized against "known dye content" as indicated by the Kjeldahl method.

Titration with Naphthol Green B

The interaction of basic and acid dyes, with the formation of relatively insoluble dye complexes has been utilized for analytical purposes by numerous analysts. The customary technic is volumetric. The dye under investigation is titrated with a solution containing a known concentration of a dye of decidedly different color and of opposite chemical type, and the end point of the reaction recognized by means of "spotting out" the solution on filter paper. This method was preferred above all others by Pelet-Jolivet[3] in his investigations on dyeing. Its success is dependent in a large measure upon the selection of suitable dyes as precipitants.

In the present investigation naphthol green B was found a much better precipitant for neutral red and the pyronins than any other of a large and varied selection of acid dyes. Even with this dye, however, it was found necessary to modify the customary technic of the titrations to obtain satisfactory results. The approximate volume of the naphthol green solution required for the titration was determined in the usual manner by spot test. Subsequent precipitations were then carried out with small variations in the volume of acid dye solution added, and the filtrates from these were saturated with salt and again filtered. In this procedure the basic-acid dye complexes which are sufficiently soluble in water to obscure the end-point of the reaction were salted out and any excess of either acid or basic dye rendered apparent.

The Iodimetric Method

Basic dyes, in general, yield insoluble complexes with iodine. It has been shown that the dye content of methylene blue solutions may be determined with satisfactory precision by precipitating the dye with standard iodine solution and determining the residual iodine in the filtered combined solutions. The iodine absorption by the dye is dependent, primarily, upon the residual concentration of iodine in the combined solutions, and it is necessary, accordingly, to standardize the method over a range of dye concentration such as may be met in its application.[4]

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[3] Pelet-Jolivet, L. Die Theorie des Farbeprozesses. Dresden, 1910.

[4] Holmes, W. C. The iodimetric evaluation of methylene blue. J. Assoc. Off. Agric. Chemists, 10, 505. 1927.

In the present investigation this method was found unpromising with pyronin G, owing to the physical characteristics of the dye-iodine complex, which rendered it difficult, or impossible, to effect a complete separation of precipitated dye. Satisfactory results were obtained, however, with neutral red and pyronin B. Particular attention was given to avoiding any appreciable loss of iodine in the filtration, and it was found necessary to conform rigidly to a precise technic to avoid appreciable variations in results.

The Spectrophotometric Method

The spectrophotometric method of dye analysis depends upon the fact that the extinction coefficients of dye solutions, under suitable conditions, vary in direct proportion with dye concentrations.

The most important factor in the spectrophotometric evaluation of dyes is the selection of the most favorable solvent. Each of the dyes under investigation undergoes tautomeric alteration in aqueous solutions, with variation in dye concentration,[5] and each is adsorbed by the glass of containing vessels in some degree. The employment of an aqueous-alcoholic solvent stabilizes the dyes and minimizes adsorption. In the instance of neutral red it is advisable to make the solvent definitely acid in order to render the dye stable also to minor variations in hydrogen-ion concentration and to convert it into the form in which its light absorption may be measured most accurately.

The following technic was adopted: With the pyronins 1.0 g. samples were dissolved in a liter of distilled water, 5.0 cc. aliquots then further diluted to 250 cc. with water and this second solution then diluted with an equal volume of 95% alcohol. The extinction coefficient of a 1.0 cm. layer of the final solution was measured with a Bausch and Lomb spectrophotometer of the latest type at 550mμ, in the instance of pyronin G, and at 555 mμ in that of pyronin B. The same procedure was employed with neutral red with the exception that 5.0 cc. of glacial acetic acid was added in the course of the dilution of the aliquot and that the adsorption measurements were carried out at 540mμ.

Comparison of the Four Methods

Following the standardization of these various methods they were tested in the examination of a number of samples of neutral red and pyronins of biological stain grade. It was found that satisfactory checks could be obtained in duplicate analyses of any sample by any individual method, but that the relative dye contents of different

[5]Holmes, W. C. The influence of variation in concentration on the absorption spectra of dye solutions. Ind. & Eng. Chem., 16, 35. 1924.

samples of the same dye as indicated by different methods varied. The variations observed between different chemical methods were relatively small, but those observed between chemical methods in general and the spectrophotometric method were frequently considerable.

The diversity in dye content values, as indicated by the Kjeldahl and spectrophotometric methods, is illustrated by the data of Table 1. For purposes of comparison the actual percentages of dyes in the samples, as determined subsequently, are included in the final column.

TABLE 1. DYE CONTENT VALUES BY VARIOUS METHODS.

Sample	Per cent N Kjeldahl Method	Extinction Coefficient	Indicated Dye Content Kjeldahl Method	Indicated Dye Content by Spectrophotometer	Actual Dye Content
Neutral red (Cass.) Std...	10.00	.775	51.3	51.3	46.1
" " (NX-1).....	15.39	1.11	79.0	73.4	66.1
" " (NX-3).....	15.465	1.175	79.4	77.7	69.9
" " (CX-1).....	10.03	.785	51.5	51.8	46.7
" " (CX-2).....	8.405	.705	43.15	46.3	42.0
Pyronin B (NP-3) Std....	5.27	1.215	67.25	67.25	33.0
" " (NP-2).....	5.555	1.18	71.15	65.2	32.1
" G (I.F.G.A.) Std.	1.13	.36	12.2	12.2	11.4
" G (Hoechst)....	1.51	.45	16.3	15.2	14.0
" G (Grübler).....	1.26	.43	13.6	14.6	13.7

Such discrepancies in analytical results indicated clearly the presence of interfering substances. When a careful spectrophotometric examination of different samples of the same dye failed to reveal the presence of any appreciable proportions of subsidiary coloring matter it appeared evident that the interfering impurities in question were colorless dye intermediates. This supposition was substantiated when the standard samples were recrystallized and reexamined. The recrystallized samples absorbed light in precisely the same manner as the original samples, but the degree of absorption in proportion to the dye content as indicated by the nitrogen values increased materially.

It was obvious, accordingly, that market supplies of these dyes contain residual dye intermediates which interfere with the determination of their dye content by chemical means. Such intermediates invalidate results obtained by the Kjeldahl, iodimetric and naphthol green titration methods respectively, because they, as well as the dyes, contain nitrogen, absorb iodine and react with and are precipitated by naphthol green. They do not interfere with the spectro-

photometric method, however, since they have no appreciable absorption of light in the visible spectrum.

Of the indicated dye content values recorded in Table 1, accordingly, those calculated from nitrogen determinations are without either absolute or relative significance. Those calculated from spectrophotometric readings afford a reliable index of relative dye content between samples of the same dye, but are worthless in the absolute sense as the method was originally standardized against erroneous dye content values.

The Method for Determining Actual Dye Content

It was evident, then, that it would be necessary to rely upon the spectrophotometric method for the analysis of such impure grades of neutral red and of the pyronins as are found on the market, but that the spectrophotometric method could not be standardized against such products. For the latter purpose it was essential to obtain dye samples of known dye content. This implies sufficient purification from nitrogenous dye intermediates to enable the analyst to rely upon dye content values determined by the chemical (Kjeldahl) method.

With this end in view samples of the dyes were repeatedly recrystallized from aqueous solution, analyzed by the Kjeldahl and spectrophotometric methods, and their apparent specific extinction coefficients calculated. The specific extinction coefficient of the dye may be defined as the extinction coefficient which it would give in the technic which has been previously outlined in the description of the spectrophotometric method provided the sample analyzed was pure dye. The same value would also be obtained, irrespective of the purity of the sample, if the observed extinction coefficient were divided by the true dye content of the sample expressed in decimal notation. The apparent specific extinction coefficient is the corresponding value obtained by employing the apparent dye content of the sample as indicated by chemical analysis.

In the present instance, in which impurities are present which introduce a positive error in the determination of dye content by chemical means, it is evident that the apparent specific extinction coefficients of the impure dye samples will be lower than their true specific extinction coefficients. The values will increase, however, with each successive step in the elimination of nitrogenous dye intermediates, and will reach a constant maximum eventually. At this point the analyst will be justified in assuming that intermediates have been eliminated (or reduced to a negligible minimum), that the apparent specific extinction coefficient is the true specific extinction

coefficient and that it may be relied upon for subsequent analytical application.

Certain of the data of the purification of the dye samples in the present investigation are recorded in the following table:

TABLE 2

Sample	Per cent N by Kjeldahl Method	Dye Content Calculated from N Value	Extinction Coefficient	Apparent Specific Extinction Coefficient
Neutral red—original.....	10.00	51.3	.775	1.51
" " —1st recryst....	14.66	75.3	1.19	1.58
" " —2nd recryst...	14.855	77.8	1.30	1.67
" " —3rd recryst....	16.61	85.3	1.43	1.68
Pyronin B—original.....	5.27	67.25	1.215	1.81
" B—1st recryst.....	6.12	78.4	2.73	3.48
" B—2nd recryst.....	6.08	77.8	2.87	3.69
" B—3rd recryst.....	5.99	76.7	2.81	3.66
" G—original.....	1.13	12.2	.36	3.12
" G—1st recryst.*....	6.89	74.4	2.35	3.16

*Insufficient material was available for further recrystallization.

Analytical values obtained with a sample of neutral red iodide of laboratory preparation, received from Dr. Max Phillips,[6] and a sample of twice crystallized pyronin G iodide, prepared at this laboratory, indicated apparent specific extinction coefficients for those dyes, respectively, of 1.64 and 3.13, calculated to the dye chloride basis.

It was concluded that the true specific extinction coefficients of neutral red, pyronin B and pyronin G are, respectively, 1.68, 3.68 and 3.15, and the employment of these values is recommended in the analysis of the dyes by the spectrophotometric method.

The spectrophotometric method of analysis is extremely convenient in application. The absorption of the dye sample is measured in accordance with the technic outlined in the previous description of the method, and its dye content found by dividing its observed extinction coefficient by its specific extinction coefficient. To obtain the dye content of a sample of pyronin B, for example, it is only necessary to prepare a 0.01% solution in a mixture of equal parts of water and alcohol, measure its absorption at 550 mμ in a 1.0 cm. layer, and divide the observed extinction coefficient by 3.68.

Acknowledgment: The authors are indebted to R. Walker of the Bureau of Chemistry amd Soils for valuable assistance in the determination of nitrogen values of various dye samples.

[6]Phillips M. and B. Cohen. The preparation of neutral red iodide suitable for biological staining. Stain Techn., 2, 17. 1927.

A GELATIN FIXATIVE FOR PARAFFIN SECTIONS

ARTHUR W. HAUPT, *University of California at Los Angeles*

Mayer's albumen fixative, of which the active principle is white of egg, is used almost universally for affixing paraffin ribbons to the slide. About eight years ago the writer's attention was called to a gelatin fixative which has proved to be so superior to albumen that he has used it almost exclusively ever since in the making of a great variety of botanical preparations, and has recommended it to a number of other workers whose experience with it subsequently has been just as satisfactory. The gelatin method was first described by Szombathy[1] and later discussed by Artschwager,[2] but it does not seem to have received the attention in the literature which its importance deserves. It certainly merits a wide spread use among both botanists and zoologists.

The gelatin fixative is prepared as follows: Dissolve 1 g. of gelatin in 100 cc. of distilled water at 30° C. Then add 2 g. of phenol crystals and 15 cc. of pure glycerin. Stir well and filter. The original formula calls for 1 cc. of a 2% solution of sodium salicylate instead of the phenol, but we have found that phenol is a more satisfactory preservative, especially against the growth of molds. Only the best grade of gelatin should be used, and the temperature should not be allowed to exceed 30°. Gelatin which dissolves at a higher temperature is not satisfactory. If a day or two is required for the gelatin to dissolve, decomposition will probably begin, and if the phenol is added too soon the undissolved gelatin will turn white and harden.

As in using albumen, the slide must be clean before mounting the ribbon upon it. Place a small drop of gelatin fixative on the slide and smear with the finger so that only a thin film is present. Flood with a 2% solution of formalin in distilled water. Mount the ribbon on the slide and warm slightly on a copper plate to straighten it. Then pour off the excess formalin solution, and allow the slide to dry for at least 12 hours at room temperature, or place in an oven at 30°. In working with material which has a tendency to become loose from the slide, place a small uncovered dish of concentrated formalin in the oven with the slides. The formalin renders the gelatin insoluble and thus holds the sections to the slide.

[1]Szombathy, K. Neue Methode zum Aufkleben von Paraffinschnitten. Zts. Wis. Mikr., **34**, 334–336. 1918.

[2]Artschwager, E. F. A new fixative for paraffin sections. Bot. Gaz., **67**, 373–374. 1919.

The method of using the gelatin fixative, as described above, follows the schedule of Artschwager, and produces more satisfactory results than the methods originally employed by Szombathy. At least, that has been the writer's experience.

Mayer's albumen fixative does not keep well, and so must be freshly prepared at frequent intervals. Gelatin fixative seems to keep indefinitely; we are still using some prepared four years ago. Albumen is colored with most of the stains in common use, producing an unsightly matrix around the sections. This is a decided disadvantage in such work as staining bacteria in soil films.[3] Gelatin is not stained by any of the anilins or hemateins. Dust particles which alight on the slides while drying are readily held by albumen, but not by gelatin.

The most serious disadvantage of albumen fixative is that often it will not hold sections to the slide, especially thick sections and those of hard material. In many such cases, where albumen fails gelatin will produce perfect results, especially if the slides are exposed to formalin vapor while drying, as described above.

[3] A gelatin fixative for soil films was in fact suggested in 1917 by H. J. Conn, The direct microscopic examination of bacteria in soil. Abstr. Bact. 1, 40.

BASIC FUCHSIN FOR STAINING VASCULAR BUNDLES

J. H. GOURLEY, *Dept. of Horticulture, Ohio Agricultural Experiment Station, Wooster, O.*

The following procedure has been found helpful in tracing the vascular supply of succulent plants, such as the garden pea, *Pisum sativum*. It has been used as a supplementary means of study only, to microscopic slides cut serially thru the plant.

In selecting a stain for this purpose it is necessary to have one that will stain the vascular bundles and fibers without diffusing thru the parenchymatous tissues; also it must remain in these tissues when the plant is run thru alcohols to clear it. Of the stains used in this work basic fuchsin (not acid fuchsin) proved most satisfactory and answered every requirement of the study.[1] This solution was prepared as follows: 50 mg. basic fuchsin, dissolved in 2 cc. 95% alcohol and diluted with 100 cc. tap water. Young or old plants of the garden pea, sweet pea, stock, calendula, tomato, etc. were lifted from the soil and the roots washed free of adhering material. The root system was immersed in the staining solution and a part cut off beneath the surface of the solution. The plants were left in this solution for from 24 to 48 hours when the vascular bundles and fibers were found to be well stained, even to the minute ones in the leaves, stipules, and cotyledons in the case of young plants. This is true even with full grown plants, measuring some six feet in height. Sometimes it seems necessary to recut the roots since some plants fail to become stained.

Upon removal, the roots were washed with water and then with dilute alcohol to remove the adhering stain thoroly. The entire plant, or parts thereof, were then treated in either of two ways, depending upon the procedure of study. In one case they were boiled in water or very dilute solution of KOH. After partial disintegration they were dissected under a binocular dissecting microscope. This allowed one to separate easily the larger vascular units and follow their general course.

Instead of dissecting the treated plants they can readily be cleared by placing in a large test tube or other container and run thru 50, 60 70, 85, 95% alcohol and absolute alcohol. Next the solutions are changed as follows: ¾ absolute alcohol and ¼ xylol, ½ alcohol and ½ xylol, ¼ alcohol and ¾ xylol, and finally in pure xylol. A period

[1]Two lots of basic fuchsin were used in this work, both purchased some time ago, one from the Will Corporation the other from Coleman and Bell, and neither lot certified by the Stain Commission.

of 10 or 12 hours or more intervened between the changes of solutions indicated above. After the plants have cleared they can be most conveniently examined under a binocular dissecting microscope of low power (eye pieces 15×, objectives 7×), with the aid of an intense light focused on the object.

In the case of the plant which was studied in particular (*Pisum sativum*) it was quite possible to follow the triarch arrangement of the roots, the main stellar and cortical bundles, and those of the cotyledons, leaves and stipules. Detailed study was, of course, necessary by means of prepared and stained microscopic slides. The method would seem, however, to have value in many types of studies, where the general arrangement of vascular strands is desired.

NOTES ON A COMBINED PEROXIDASE WRIGHT'S STAIN FOR BLOOD FILMS

ARTHUR T. BRICE, JR., *McLeod Infirmary, Florence, S. C.*

Washburn[1] has described a combined peroxidase reaction and Wright's stain for blood smears, making use of a modification of the original Goodpasteur formula. Strumia[2] has described a peroxidase reaction technic for blood smears and frozen sections in which the basic fuchsin of the original Goodpasteur formula is eliminated. It occurred to us that the fuchsin might be omitted in the combined method described by Washburn, thus eliminating one reagent and shortening the technic by avoiding the necessity of the decolorizing step. The use of the fuchsin in the original Goodpasteur stain would seem to have been solely for the purpose of giving the non-peroxidase reacting cells a color and its use in a technic, the purpose of which is to bring out the Wright's staining characteristics of the cells as well as the peroxidase characteristics, would therefore be superfluous. We have tried this experiment and found it to be entirely successful. In our technic for producing a combined peroxidase-Wright's stain the following solutions are used:

Oxydase Reagent—Dissolve 0.3 grams of benzedine base in 100 cc. of 95% ethyl alcohol. Add 1 cc. of a saturated aqueous solution of sodium nitroprusside. This reagent should keep equally as well as the Beacom modification of Goodpasteur's stain, namely 8 months to a year.

Hydrogen Peroxide—1/200 solution made by adding 2 to 3 drops to 15 cc. of distilled water. This must be freshly prepared immediately before use.

Wright's Stain according to the usual formula.

Thin films should be made and dried in air. The slide or cover slip is first flooded with the oxydase reagent which is allowed to act for from one to one and one half minutes. An approximately equal portion of the hydrogen peroxide solution is then poured on the slide and allowed to act for three minutes. The slide is then washed thoroly under the tap and dried by blotter and in air. The drying is necessary in order to remove the water from the film to permit of its being stained by an alcoholic stain.

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[1]Washburn, A. H. A combined peroxidase and Wright's stain for routine blood smears. J. Lab. & Clin. Med., **14**, 246. 1928.

[2]Strumia, M. M. A modification of the peroxidase reaction, etc. Arch. Path. **5**, 447. 1928. Cited from J. Lab. & Clin. Med., **14**, 1204. 1929.

The film is then stained by Wright's method according to the usual technic and employing normal time factors. The resultant stain with normal blood gives a very clear cut picture of the differential characteristics of the cells. Peroxidase granules stand out sharply. In the polymorphs the reaction seems to be so intense as to almost disrupt the cell when freshly prepared oxydase reagent is used, the nucleus being almost obscured by them and appearing as only faintly stained by blue. The lymphocytes appear as by Wright's stain alone. Other normal cells appear as described by Washburn, the eosinophiles and basophiles being difficult of differentiation but showing characteristic large black granules which are more refractile and round than those of the polymorphs and having pale centers, the granules of the basophile being distributed more thickly about the edges of the cell and its nucleus being a paler blue than that of the eosinophile.

Washburn has found it necessary to increase the staining time by Wright's very considerably beyond the normal time for lucaemic bloods. We do not see why this should be so and suggest that it may have been due to the action of the fuchsin in his peroxidase reagent and the consequently necessary decolorizing step. At the present writing we have not yet had the opportunity to examine any lucaemic blood by this method.

A NEW METHOD FOR SAFRANIN DIFFERENTIATION

HSU-CHUAN TUAN[1], *Dept. of Botany, University of Pennsylvania, Philadelphia, Pa.*

ABSTRACT: **A new method of differentiating safranin in pollen-mother-cell smears and paraffin sections is described in detail. Slides stained in safranin are dehydrated in a series of alcoholic solutions containing 1.5% picric acid with constantly decreasing percentages of water. Differentiation is principally effected in 83% alcohol containing 1.5% picric acid and completed in the final dehydration and clearing. Counterstains may be applied in clove oil if desired.**

To every botanist and zoologist, safranin is always a valuable stain; it is usually employed, however, with some uncertainty, on account of its liability to poor differentiation for critical preparations. With this idea in mind, the author worked out the following method for differentiating safranin in pollen-mother-cell smears and in paraffin sections.

Only saturated aqueous solutions of two kinds of safranin were used, as the alcoholic solutions are unsatisfactory for this purpose. A German sample, Grübler's safranin O, and an American product, safranin O, National Aniline & Chemical Co., certification No. NS-5, did not show much difference in their staining action toward smears, but for paraffin sections of root tips, the latter was, as a rule, a little slow in registering its effect. Fortunately, this is not the important factor with which this method will deal. The distinctive feature of the method is to dehydrate and differentiate the preparations in a series of alcoholic solutions of picric acid in a gradual manner so that shrinkages produced by hasty procedures are avoided. For *Gasteria* smears, it is especially true that a 5 minute stay in each of the alcohols employed is not too long. In one lot of slides, for instance, ten grades of alcohols, at 5 minute intervals, were used; the result was very good. In other words, if a slow dehydration is desired the grades of alcohols may be increased and the time in each one of them can be conveniently adjusted to suit the situation. The author was able to obtain excellent permanent mounts of *Gasteria* pollen-mother-cells showing meiosis in 1 to 2 hours after starting with freshly collected material.

[1]Fellow of the Chinese Educational Mission at Washington, D. C. of Tsing Hua University, Peiping, China.

Method for Smears

Anthers of *Gasteria* are smeared and fixed on previously cleaned slides according to Taylor's technic.[2] After a brief washing and bleaching, the slides are transferred singly into a saturated aqueous solution of safranin for 5 to 10 minutes, since the following dehydration procedure requires a constancy of attention which makes it impossible to handle a number of slides at one time. After the first slide has been stained and transferred to alcohol, another can be placed in the stain. Thus a succession of slides may be handled and well spaced at convenient intervals in the process. The slides are rinsed in water for a few seconds to remove the superfluous safranin, then dehydrated thru a series of alcoholic picric acid solutions, differing in the amount of water present. In 10% alcohol the solubility of picric acid is less than 1.5% at room temperature, while its solubility increases as the percentage of alcohol increases, therefore a saturated solution of picric acid in 83% or 95% alcohol is too strong for this purpose. From the author's experience, a 1.5% solution is of the proper concentration.

The safranin will begin to differentiate first slowly in the lower alcohols and then faster in the higher ones. The color of the cytoplasm at this stage is deep orange, while the chromatin is much darker in intensity. When the slides reach 95% picric alcohol, the yellow color of the acid is removed by a solution of ammonia with 0.5 cc. or less of ammonia-water in each 100 cc. of 95% alcohol. If the slide passes from the picric alcohols without ammonia, the cytoplasm remains yellow and occasionally orange. For mere counterstaining this may be desirable, but the permanency of the safranin in the presence of picric acid is untested and, therefore, the ammonia used here is to insure the keeping quality of the preparation. The absence of the yellow color may be indeed preferred. The slide is then rinsed and dehydrated thru pure 95% and absolute alcohols. In fact, two absolute alcohols can be used at this point to assure thoro dehydration and removal of excess ammonia. As soon as the slide is in xylol or clove oil, the dehydration and differentiation are completed.

During the author's experimentation, various controls were made for comparison. Two of them are worth mentioning here. 1) Almost without exception, slides stained in safranin for more than one hour are muddy in appearance under the microscope. The cytoplasm is purple and the chromosomes do not stand out as sharply as they should. 2) The dehydration must go thru all the picric alcohols,

[2]Taylor, Wm. R. The smear method for plant cytology. Bot. Gaz., **78**, 236–238. 1924.

otherwise the same defect will be found. If, for instance, slides are stained in safranin for 10 minutes and then dehydrated thru pure alcohols and differentiated in 95% picric alcohol or other acid alcohols the result is not so good. The following schedule may be helpful to those who wish to make use of this method:

1. Pollen-mother-cells are smeared and fixed after Taylor's method.
2. Wash in running water for 5 minutes.
3. Bleach in hydrogen peroxide solution for 5 minutes under strong light (the solution is made up of 1 part of hydrogen peroxide and 2 parts of water).
4. Wash in running water.
5. Stain in a saturated aqueous solution of safranin for 5 to 10 minutes.
6. Rinse in water to drain off the safranin on the slide.
7. Dehydrate thru the alcohol series in the following manner:
   a. 10% alcohol saturated with picric acid for 2 minutes.
   b. 30%, 50%, 70%, 83%, 95% alcohols with 1.5% picric acid in each grade, for 2 minutes each.
   c. 95% alcohol with 0.5 cc. or less ammonia water in each 100cc. of 95% alcohol for 30 seconds.
   d. 95% pure alcohol to rinse off the ammonia.
   e. 100% alcohol 1 minute.
   f. Another change of 100% alcohol for 1 minute.
   g. Alcohol-xylol, 5 parts of absolute alcohol and 3 parts of xylol, for 2 minutes.
   h. Alcohol-xylol, 3 parts of absolute alcohol and 5 parts of xylol, for 2 minutes.
   i. Xylol 5 minutes.
   j. Mount in xylol-balsam.

Method for Paraffin Sections

What has been said about the smears is true about the paraffin sections also, except that the paraffin sections take longer for the safranin to act and the stain is usually differentiated more rapidly in the alcohols. As a rule, when slides are stained 30 minutes in any one of the safranins and followed by rapid dehydration, the differentiation is excellent and there is no way to distinguish between the staining action of these two dye samples. If the dehydration is carried on slowly, allowing 4 minutes for each grade of the alcohols, the safranin NS-5 is washed entirely from the sections, while Grübler's stain is still held in some metaphase and anaphase figures of the dividing cells. Again, if the slides are treated with dilute HCl before

safranin NS-5 is applied, the difference is not so striking (2 drops of HCl in 100cc. of water). Thus the author is able to produce the same stage of differentiation by treating the slides with a little acid before safranin O, NS-5, is applied. Generally, slides stained from 30 minutes to 3 hours give the best result, while those stained over night are, invariably, muddy or no good at all. The following two schedules may be used in parallel for two stages of differentiations.

Schedule A for darker stain:

1. Stain the slides in saturated aqueous solution of safranin for 30 minutes to 3 hours (if the dye is slow in its action like lot NS-5, treat the slides with dilute HCl before using).
2. Rinse in water for a few seconds.
3. Dehydrate thru 10%, 30%, 50%, 70%, 83%, and 95% alcohols containing 0.5% picric acid, allowing 5 to 10 seconds for each grade. If intermediate alcohol stages are desired they may be introduced and the time in each stage must then be reduced accordingly.
4. Rinse in pure 95% alcohol with or without ammonia for 10 minutes.
5. Clear in clove oil and counterstain if desired.
6. Rinse in xylol 15 minutes.
7. Mount in xylol-balsam.

Schedule B for lighter stain, especially for metaphases:

1. Stain in safranin as in "A".
2. Rinse in water for a few seconds.
3. Dehydrate thru 10%, 30%, 50%, 70%, 83%, and 95% picric alcohols allowing 1 minute for each grade. If intermediate alcohol stages are desired they may be introduced and the time in each stage must then be reduced accordingly.
4. Dehydrate in 100% alcohol for 1 minute.
5. Clear thru mixtures of xylol and absolute alcohol.
6. Wash in xylol for 10 minutes.
7. Mount in xylol balsam.

The Grübler's dye gave somewhat better results by this second schedule than did the American dye.

It is interesting to mention some observations on the mixtures of safranin and picric acid. When a very much diluted solution of safranin is poured into a solution of picric acid, the mixture becomes orange in color. If a concentrated safranin is used, a red flocculent precipitate is immediately formed. This precipitate, if washed and filtered, is practically insoluble in water and aqueous picric acid,

but slightly and sluggishly so in alcoholic picric acid and readily in alcohol alone. It seems that this indicates that the stain remaining in the chromatin after the treatment with picric acid is of this nature rather than safranin alone. On this assumption, the author tried the alcoholic picro-safranin precipitate on some smears and root tip sections. The result for root tip sections, so far, is of no value for both the cytoplasm and the chromatin take up the stain equally and no differentiation was obtained; moreover, the remaining color is far from brilliant but rather suggests the purplish or muddy cytoplasm described in a foregoing paragraph. In the smears, if the dehydration is fast enough, it stains the chromatin light red. But it is clear that in this process of dehydration, safranin combines with the picric acid to form a substance, which, due to its peculiar solubility in picric alcohol, is chiefly responsible for the success in differentiating the safranin.

Searching in the literature it is found that Lōwit[3], years ago recommended the use of a mixture of tincture of iodine and picric acid to rinse the sections of blood-cell producing organs for 5 to 20 seconds before clearing in clove oil and mounting. The mixture consisted of 3 to 5 cc. of 1% alcoholic picric acid and 1 to 2 drops of tincture of iodine. When root tip sections of plants are thus treated, the cytoplasm will retain a brownish coloration which is, of course, undesirable for cytological purposes. Recently, Haynes[4] succeeded in differentiating several samples of safranin by means of fast green, and Katznelson[5] obtained a polychrome effect on animal tissue, by means of safranin and picric-indigo-carmin. It is the author's conviction, that the use of picric acid in plant cytology can be widened further to some other basic nuclear stains. For instance, with malachite green, he obtained quite a number of good results on onion root tip sections. With patience and intensive experimentation, many other stains may be thus employed with satisfaction.

[3]Lōwit, M. Die Anordnung von Leukoblasten und Erythroblasten in den Blutzellen blidenden Organen. Arch. f. Mikr. Anat., **38**, 524–612. 1891.

[4]Haynes, R. Fast green, a substitute for light green SF yellowish. Stain Techn., **3**, 39–40. 1928.

[5]Katznelson, Z. S. Über die Methodik der Safraninfärbung. Zts. Wis. Mikr., **46**, 177–189. 1929.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

BAILEY, H. D. A flagella and capsule stain for bacteria. Proc. Soc. Exper. Biol. and Med., **27**, 111–2. 1929.

1. A small amount of material from a 15 to 24 hour agar growth is diluted thru two successive drops of water on a slide and a loopful of the second is smeared on a clean slide. Dry without heat.
2. Treat with mordant (5% tannic acid 3 parts, 10% $FeCl_3$ 1 part, preferably filtered before use) 2 minutes. Mordant will keep indefinitely.
3. Mix 7 drops of mordant with 1 drop Ziehl-Neelson carbol-fuchsin. Add 1 drop concentrated HCl, and mix. Add one drop formaldehyde (40%?), and mix. Cover smear with this mixture freshly prepared after pouring off mordant; allow to stand 7 minutes.
4. Wash in running water.
5. Apply Ziehl-Neelson carbol fuchsin and steam gently 30 seconds. Staining fluid may be a mixture of this with anilin gentian violet if more intense staining is desired. Stain should preferably be filtered before use.
6. Wash in running water.

Method may be used for staining exudate from white mice killed with pneumococci, as follows: 1) Mix loopful exudate with loopful water and make thin smear. 2) Apply mordant 10 seconds. 3) Wash. 4) Apply cold diluted carbol fuchsin 10 seconds. 5) Wash, blot, and examine.

CARTWRIGHT, K. ST. G. A satisfactory method of staining Fungi mycelium in wood sections. Ann. Bot., **43**, 412–413, 1929.

This is a relatively quick and simple method by means of which the finest hyphae are revealed in the wood. It proves successful with some representatives of the following groups of fungi: Basidiomycetes, Ascomycetes, Fungi Imperfecti and molds.

1. Stain in 1% aqueous safranin (usually a very short application is sufficient) and wash in water.
2. Flood the sections with a mixture of 25 parts saturated aqueous anilin blue and 100 parts saturated aqueous picric acid, and heat over a flame until it begins to simmer. Wash.
3. Run up to 100% alcohol, clear in clove oil, mount in balsam.

The preparation should show red lignified walls and clear blue mycelia. Areas where the wood is badly decayed may appear bluish but the hyphae are always well defined.

CLAUSER, F. and STRANI, M. Primäre Färbung durch Chloroform und saure Farbstoffe. Zts. Wis. Mikr., **47**, 58–63. 1930.

The author finds that chloroform facilitates nuclear staining of unfixed tissues with acid and electro-negative colloidal stains. The staining is almost instant provided that only two substances (chloroform and the stain) are used. Intensity of the stain depends upon its concentration, the time factor being of little importance.

Three groups of stains were applied: a) Electro-negative colloidal stains (acid or better anodical vital stains): trypan blue and carmin. Without the chloroform they give a faint nuclear stain after prolonged action and fail to stain fatty tissue. b) Basic: thionin, gentian violet and bismarck brown. These stain also without chloroform, but the subsequent dehydration and xylol treatment are detrimental. c) Acid: eosin, orange G, methyl blue. These fail to stain at all without chloroform. (Source or dye content of the stains is not mentioned.) All above stains were used in 1% aqueous

solution, 3 cc. of which were mixed with 1 cc. of chloroform or ether. Rabbit's omentum stained 5 minutes showed clear nuclei and presented pictures similar to sections treated in the usual way. The method may also be used on live animals. It proved successful by injections in various organs. The chloroform staining method does not change the physico-chemical properties of the sections and they may be subsequently counterstained.

DAVENPORT, H. A. Silver impregnation of nerve fibers in celloidin sections. Anat. Rec., **44**, 79–83. 1929.

The following method proved successful with formalin fixed mesencephalon of cat and material of human autopsy, except for the cerebral cortex.

1. Cut celloidin sections 15 to 30 μ thick and transfer to 80% alcohol.
2. Place 1 to 4 hours (cerebellum and brain stem seem to require less time than the spinal cord) in mixture of 85 cc. 95% alcohol with 15 cc. concentrated nitric acid.
3. Wash in 3 to 4 changes of 80% alcohol.
4. Allow to stand at 37° to 40°C preferably over night in silver nitrate bath (10 g. dissolved in distilled water and added to 90 cc. 95% alcohol). For gold toning impregnate 18 to 24 hours.
5. Rinse in absolute or at least 95% alcohol.
6. Develop 3 to 5 minutes in the following: 3 g. pyrogallic acid, 95 cc. 95% alcohol, 5 cc. neutral formalin (40%). If too dark, place 1 to 15 minutes in alcohol before developing and develop only 1 to 2 minutes. If too light omit alcohol or pass thru it very rapidly, and develop 10 minutes or more.
7. Clearing and toning by any of following methods: a) pass thru 50% alcohol, fix until chocolate brown, 10% aqu. sodium thiosulfate (or use ordinary photographic fixing bath, Eastman Kodak Co's formula F-1 diluted with water 1:1); wash, dehydrate and mount. b) 2 to 3 changes of 80% alcohol, 95%, absolute alcohol, xylol, balsam. c) Gold toning by any of the standard procedures; in which case development should be longer.

One must impregnate in the dark, but the rest of the handling may be done in diffuse light. Brush off precipitate before mounting. Application of an alcoholic instead of aqueous silver nitrate and of alcohol instead of water for rinsing improves the staining of the fibers. The pretreatment with alcoholic nitric acid inhibits the staining of glia and other connective tissue fibers, and thus assures a selective stain for fibers in celloidin sections.

DUFF, D. C. B. A modification of the Orskov single-cell technic. J. Lab. & Clin. Med., **15**, 186–188. 1929.

The original Orskov technic for isolating pure cultures of bacteria arising from single cells is essentially as follows: A microscopic slide is scored with fine lines with a diamond pencil and is sterilized; a block of agar on which a dilute culture of the organism under investigation has been smeared is placed on the slide; a single cell is found by microscopic examination, its location on the mechanical stage noted, and a sketch made of the lines on the slide intersecting in its neighborhood; the slide is incubated under proper moisture conditions for a few hours, and is returned to the microscope; the original cite is located and the colony produced from the single cell noted; the objective is then replaced with another on which a sterile needle has previously been mounted with wax; this needle is then lowered onto the colony, withdrawn, and the growth adhering to it washed off with a drop of sterile culture fluid. The writer modifies this technic by using the Chambers' micro-manipulator so as to have the colony under observation at the moment of isolation. After locating the colony as above described a sterile pipette mounted in the micro-manipulator is touched to the colony while the latter is under observation. The pipette is then withdrawn and the tip broken off into about 2 cc. of sterile medium. The writer gives the various steps in detail and claims that the method requires no abnormal degree of technical skill.

FIALA, GRACE F. Preparation of hair for cross section examination. Amer. J. of Physical Anthropology, **14**, 73–74. 1930.

Method for gross examination of cross section of hair:

1. Get a block of white pine 1x1x0.5 cm. and hollow out the top in the line of the grain, forming a shallow groove. Cut a small notch at the middle of the edges of both sides of the groove.

2. Turn over the block and fasten a strand of hair to the bottom with sealing wax. Draw the hair around the block, guiding it thru the notches so that it is stretched over the groove. Fasten the other end in the same manner.
3. Moisten the hair above the groove with a drop composed of equal parts of absolute alcohol and ether. Apply thick celloidin to cover the hair.
4. Harden (about 10 minutes), and transfer to Gilson's mixture (equal parts of chloroform and cedar oil). Add cedar oil every half hour until it composes 90%. Remove and dry in the air, few minutes.
5. Cut thru both hair and block with a sharp knife or razor.

By standing half of the block on its side on the condenser of the microscope, the cut ends of hairs can be seen by reflected light as dark ovals and circles against a light background. The block may be preserved in a dry stoppered bottle.

SATO, KUNIO. On the real nature of vital staining. Folia Anat. Jap., **8**, 51–61. 1929.

Vital staining is due to dye particles taken up in the cell body by a physical process. The density of the tissue elements and the diffusibility of the stain play a most important role in staining. Potassium compounds loosen tissue elements while calcium condenses them.

Trypan and toluidin blue and neutral red (source and dye-content not mentioned) were used in aqueous solutions, carmin in the form of lithium carminate. Nothing new was found with these stains commonly used for vital staining. India ink is taken up only by cells capable of phagocytosis by the usual technic. When mixed with potassium compounds more and larger granules are formed by all phagocytes: in the kidney it is found in wandering cells of histiocytes in the glomerular capsules and of connective tissue; in the liver, in stellate cells and histiocytes of the interlobular connective tissue and even in the parenchym cells, (which do not take India ink except in presence of potassium salts); in the spleen India ink appears in endothelial cells of the spleenic pulps and in the spleenocytes as large granular masses, while traces are found in the reticulo-endothelial cells of spleenic corpuscles. When mixed with calcium compounds India ink is not taken up by such cells as the liver parenchym nor the reticulo-endothelial cells of the spleenic pulp. Generally granular masses ingested in the phagocytes are rare and small. Acid fuchsin, eosin, orange yellow are diffusible stains which usually fail to deposit visible granules. Potassium makes the dye stain more faintly while calcium intensifies it.

LILLIE, R. D. The Gram stain: A quick method for staining Gram positive organisms in tissues. Arch. of Path. and Lab. Med., **5**, 828. 1928.

The author gives a brief historical sketch of the progress in development of the Gram's technic of staining, since its introduction by Christian Gram in 1884. The Weigert method of staining is criticized adversely. Acetone was used for decolorization by Kisskalt in 1901, and later by Burke and by Kopeloff. In staining bacteria in tissues, the author has turned to the use of the Hucker ammonium oxalate crystal violet solution, employing pure acetone as the differentiating agent. Tables are given which demonstrate the results obtained for many strains of bacteria. The detailed technic employed is as follows:

Paraffin sections of about 5 microns or frozen sections fixed to the slide by the celloidin method of Mallory and Wright are brought down to water in the usual way.

1. Sections are stained for 30 seconds with Hucker's ammonium oxalate crystal violet; one minute for tubercle bacilli and leprosy bacilli; heat if necessary to shorten the time of exposure.
2. Wash in tap water.
3. Flush in Lugol's solution for 30 seconds; wash.
4. Apply acetone drop by drop for 10 to 15 seconds.
5. Wash in water, but do not dry.
6. Counterstain with 0.5% safranin for 30 seconds.
7. Wash in water.
8. Dehydrate and differentiate with acetone.
9. Clear with xylol.—*Sara A. Scudder.*

LYNCH, J. E. Eine neue Karminmethode für Totalpraparate. Zts. Wis. Mikr., **46**, 465–469. 1930.

Borax carmin has been used for staining total specimens such as large protozoa, hydroids, bryozoa, trematodes, amphioxi and chick embryos during a period of 12

years. It proves dependable, keeps well and is therefore recommended for class material.

1. Fix in either Zenker's, Schaudinn's, Gilson's, Bouin's, cobalt nitrate of Da Fano, acetic sublimate, or preferably in formol, wash (if necessary) and run up to 80% alcohol. Transfer to 50 or 35% alcohol and then to alcoholic carmin of Grenacher, (use Grübler's "rubrum opticum" carmin) for two hours, a day or more, until the specimen are well permeated with it.
2. Agitate container and drop in HCl until a brick-colored precipitate is formed. Stir well and leave over night or longer. Avoid excess acid.
3. Pour off the stain and run up thru slightly acidulated alcohols to 70% alcohol. Decolorize by adding 5% or more concentrated HCl. Wash in several changes of 80% alcohol when differentiation is complete.
4. Counterstain by adding Grübler's indulin (saturated in 80% alcohol) to the alcohol containing the material, until it appears a light blue. Transfer to 95% alcohol when the borders of thin membranes and cilia become light blue. Watch the process as indulin is hard to extract if overstained. Usually apply a few minutes to one hour.
5. Transfer to absolute alcohol, alcohol-xylol, then xylol, and mount in balsam.

Other possible counterstains are: picric acid with indulin, light green, Lyons blue, or indigo carmin.

J. C. H. de Meyere. Über haltbare rasche Färbung vermittels Azetokarmin. Zts. Wis. Mikr., **46**, 189–195. 1929.

Permanent aceto-carmin preparations may be secured by the following fast methods. For testes of insects or other animals:

1. Dissect in physiological salt solution and fix in 50% acetic acid, 15 min. for small objects.
2. Dry with filter paper, transfer and tease on a slide smeared with albumen. Leave in absolute alcohol over night.
3. Cover the material with few drops of aceto-carmin. Iron alum or ferric hydroxide may be added to intensify the staining. When stained, flood and rinse in absolute alcohol. Mount in Venetian turpentine preferably, for it tends to swell the cells and keep the chromosomes well apart, or in balsam.

For pollen mother cells:

1. Apply hot Carnoy's fluid for few minutes.
2. Transfer to slide and tease out in aceto carmin, isolate from debris and cover with a 21x26 mm. cover glass. Heat slowly to boiling, but do not boil.
3. Remove aceto-carmin with filter paper, replace it with acetic acid and then with turpentine.

The method proved successful for filamentous algae, (which may be also stained without Carnoy's fluid), and for vegetative points. If handling Infusoria cover with Carnoy's on slide, then with aceto-carmin (heating unnecessary), acetic acid and turpentine.

Okajima, K. Uber die Färbemethode mit Alizarinmolybdänlack. Folia Anat. Jap., **4**, 411–412. 1926.

Erythrocytes, blood crystals, corneous suostance, etc., stained by this method, do not fade in years even if exposed to sunlight. Eosinophile granules are also stained. The method is:

1. Fix in formalin.
2. Wash sections and mordant in a 10% aqueous solution of phosphomolybdic acid for 30 seconds to 2 minutes. Wash.
3. Stain 20 minutes to 20 hours in 100 parts of saturated aqueous sodium alizarin sulfonate mixed with 30 parts of 10 % aqueous phosphomolybdic acid. Wash.
4. Alcohol, xylol, balsam.

The aqueous solution is more permanent than the alcoholic. The staining agent is an alizarin molybdic acid lake. This lake may be obtained dry or in solution (Alizarinmolybdänlack sicc. and solut.) from Grübler & Co., Leipzig, Germany.

Owen, Helen H. and Band, Marie. The Stoltenberg differential stain for diphtheria bacilli. Am. J. Pub. Health, **20**, 426–7. 1930.

A comparison is made of the Stoltenberg (*Deut. Med. Wochnschr.* **52**, 1304. 1926)

diphtheria stain with the Loeffler alkaline methylene blue and the Albert stain. The Stoltenberg procedure is as follows: Stain 1 minute in

Malachite green	0.25	g.
Toluidin blue	0.05	g.
Hematoxylin	0.01	g.
Distilled water	100	cc.
Acetic acid (strength not stated)	3	cc.
Ethyl alcohol (95%?)	3	cc.

Wash with tap water and dry. The granules appear red and the body green.

Greater differentiation of the polar bodies is observed than with the Loeffler methylene blue. The Stoltenberg stain is not recommended, however, as a substitute for it in routine work, as it does not differentiate *B. diphtheriae* as satisfactorily from *B. xerosis* or other non-pathogenic forms with polar staining. It is recommended as a valuable confirmatory stain, nevertheless, since it aids in the differentiation of *B. diphtheriae* from *B. hofmanni*, especially in young cultures, and is a very short and simple procedure.

POLICARD, A. and H. OKKELS. Localizing inorganic substances in microscopic sections. The micro-incineration method. Anat. Rec., **44**, 349–361. 1930.

In ordinary microscopic sections of any tissue the inorganic "skeleton" within the cells remains intangible; only a few qualitative tests from analytical chemistry can be transferred to the surroundings within the cell bodies; the inorganic matter, particularly heavy metals, are "masked", which means that they probably are present in the form of complex compounds. For the histologist the method of mineralizing sections with strong mineral acids is worthless, because no structural remains are left, yet organic matter must be removed before microchemical tests can be applied.

To solve this problem the following method has been devised. Mounted sections are heated in a red-hot tube furnace in such a manner as to burn all organic compounds. The entire mineral part of the tissue remains as ashes, arranged on the slide in the same position as it occurred in the living cells, thus reproducing completely outlines and general features of the tissue. In this ash preparation any organ or tissue can be recognized.

The method can be utilized in two ways: 1) to determine the distribution of inorganic matter as a whole thruout a given section; 2) as a preliminary process before localizing definite mineral substances by means of specific reactions. In the ashes inorganic matter is now free ("unmasked") and lends itself readily to further microchemical tests, both microcrystallographic ones and those based on colors.

REES, C. W. Fixing thin blood smears for staining with iron hematoxylin and with Giemsa's stain. Science, **71**, 134–135. 1930.

Good preparation of blood smears with parasites (*Babbesia bigemina*) stained with iron hematoxylin and Giemsa's stain may be obtained in the following method: Thin smears are dried as for Giemsa's preparations and fixation completed by Schaudinn's alcoholic sublimate without acetic acid, or following wet fixation by formaldehyde. The latter is accomplished by dropping the smear into 40% formalin and then treating with alcoholic sublimate as for dry smears. The method is especially advantageous when blood of anemic cattle is used or when humidity retards drying.

RHAMY, B. W. Rhamy triple stain for frozen sections. 1. Methods for frozen sections J. Lab. & Clin. Med., **15**, 490–491. 1930.

For temporary preparations: Cut tissue into 4% formalin, float on slide, stain 10–15 seconds in:

Eosin (source not mentioned) sat. aqu. sol.	5 cc.
Basic fuchsin for bacilli (Grübler) sat. alc. sol.	3–5 cc.
Methylene blue for bacilli (source not mentioned) sat. alc. sol.	15 cc.
Grain alcohol 30%	q.s.a. 100 cc.

(This stain can be used fresh but is better after 48 hours ripening. Should be purplish blue; and when correct should stain *B. coli* violet, *B. typhosus* pink, while *B. diphtheriae* shows blue granules with pink interspaces.)

Rinse with water or dextrose solution and examine.

For permanent preparations: Cut, stain and wash as above (or dip in stain before mounting); then transfer to 96% alcohol until excess stain is removed (5 to 10 seconds). Transfer to absolute alcohol, carry thru xylol and mount in neutral balsam.

For free-hand razor sections: This stain can be substituted for polychrome methylene blue in the Terry technic. The picture is practically the same for both stains.

RHAMY, B. W. Rhamy triple stain for frozen sections. 2. Quick method for mounted sections (24 hours). J. Lab. & Clin. Med., **15**, 491–492. 1930.

The same stain proposed for frozen sections may be used on celloidin sections by the following technic:

1. Fix in 5 to 10% formalin 1 hour.
2. Two changes of acetone ½ to 2 hours each, according to size of specimen.
3. Acetone, absolute alcohol, ether (equal parts) ½ to 2 hours.
4. Absolute alcohol and ether (equal parts) ½ to 2 hours.
5. Thin celloidin ½ to 2 hours.
6. Thick celloidin overnight.
7. Mount and cut.
8. Stain sections 10 to 15 seconds in:

Basic fuchsin for bacilli (Grübler), sat. alc. sol.	3–5 cc.
Eosin (source not mentioned), sat. aqu. sol.	5 cc.
Methylene blue for bacilli (source not mentioned), sat. alc. sol.	15 cc.
Grain alcohol, 30%	q.s.a. 100 cc.

(This stain is better after 48 hours ripening. See preceding abstract.)

9. Wash and differentiate in 96% alcohol about 10 seconds.
10. Absolute alcohol, xylol, neutral balsam.

Results: Epithelial cells light blue; endothelial cells lavender and small round cells deep blue; nuclei of spindle cells lavender blue. Connective tissue fibers pink. Glandular epithelial cells sky blue. Muscle fibers rose to magenta. Mast cells dark blue with blue granules. Concretions and mucous blue, caseous material pale red. Degenerate areas pale blue to pink. Heads of spermatozoa blue, connective piece dark red, tail pink.

RHAMY, B. W. Rhamy triple stain for frozen sections. 3. For blood smears and for bacteria. J. Lab. & Clin. Med., **15**, 492. 1930.

The same stain proposed for frozen sections may be used on **blood smears,** by the following technic:

Fix smear with denaturated or other alcohol. Wash or blot. Cover one minute with following stain:

Basic fuchsin for bacilli (Grübler), sat. alc. sol.	3–5 cc.
Eosin (source not mentioned), sat. acq. sol.	5 cc.
Methylene blue for bacilli (source not mentioned), sat. alc. sol.	15 cc.
Grain alcohol, 30%	q.s.a. 100 cc.

(Preferably ripened 48 hours. See abstract above.)

Wash with tap water, blot and dry.

Results: Nuclei blue, neutrophile cytoplasm pink. Lymphocyte cytoplasm red to reddish blue. Basophiles as in Wright stain, eosinophile granules pink to opaque white. Red cells pink to orange, polychromatophilic cells red.

Bacteria in smears and cultures may be similarly stained by fixing with heat, covering ½ to 1 minute with the above staining fluid, washing and drying.

Results: Negri bodies magenta with blue granules, nerve cells light blue. *B. typhosus* and the paratyphoids stain pink. *B. coli* and *B. dysenteriae* lilac. *B. diphtheriae* pink with lilac granules and bars. *B. influenzae* and Leptothrix colorless with lilac bars and granules. Cocci ordinarily blue; meningococci lavender to pink. Spirochetes pink or lavender. Mold mycelium dark red or lavender with blue spores.

SLONIMSKI, P. Sur un procédé simple pour décortiquer les embryons des Amphibiens Urodèles. Bull. d'Histol. Appl., **7**, 44–45. 1930.

The author recommends a method which liberates amphibian eggs (in all stages of segmentation) and embryos from their albuminous envelope and membranes. Place embryo on bristol board and remove most of the gelatinous substance with a Gillette razor blade. Thus prepared, the embryos are transferred to a piece of bristol board

(smaller than the opening of the container which is to receive the specimen) that has been soaked in paraffin. Hold object with forceps and make a sharp stroke with a Gillette blade. If embryos are not liberated instantly, carefully repeat the operation. In case of injury, use Holftreten's fluid (NaCl, 0.35; CaCl, 0.01; KCl, 0.005; $NaHCO_3$, 0.01; aq. dest., 100 cc.) The paraffin coat on the cardboard prevents drying and facilitates transfer of the specimen to the liquid into which it is to be introduced.

VILKOMERSON, I. M. A new rapid paraffin method for tissue sections. J. Lab. & Clin. Med., **15**, 290–291. 1929.

Tissues are fixed, embedded, cut and stained in 3 to 4 hours. The method is efficient and the slides are permanent.

1. Fix segments 2 mm. thick for 1½ hours in wide-mouthed bottles in a mixture of 60 cc. absolute methyl alcohol, with 100 cc. acetone C.P. in which one or two iodine crystals have been dissolved. At the bottom of the bottle there should be a ½ inch layer of anhydrous copper sulfate covered by several ply of gauze, on which the tissue is placed.
2. Transfer to chloroform for ½ hour.
3. One part chloroform, 1 part paraffin, 15 minutes.
4. Melted paraffin at 56°C, 15 minutes.
5. Melted paraffin with attached vacuum apparatus, 15 minutes. Use electric water bath at 56°C near a sink with a vacuum pump attachment connected to a Woulff bottle or with a vacuum pipe system The temperature of the water bath should not exceed 1°C above the melting point of the paraffin.
6. Embed and cut as usual.

Albumen is necessary if slides are cleaned with acid alcohol to remove the grease. Ten minutes drying is sufficient when drying is carried by 37.5° and if excess water is wiped off. Absolute alcohol and acetone fix and dehydrate simultaneously. Iodine insures penetration. Copper sulfate dehydrates. The vacuum paraffin method assures better infiltration than the usual method, and there is less danger of shrinkage thru heat.

WATANABE, HORTI. Beitrage zur Färbung der Paschenschen Körperchen (Elementarkörperchen). Zent. f. Bakt., I Abt, Orig., **116**, 291–294. 1930.

Paschen's method of staining the bodies originally found in lymph, which have been named after him (Paschensche Körperchen), was with Loeffler's mordant and usually carbol fuchsin; it can be much improved by antiformin treatment after mordanting. Petragnani and Gianni's mordant for bacteria in tissues is also satisfactory providing methyl alcohol is used instead of absolute. Chromic acid also is a useful mordant. Smears from certain skin diseases served for material.

The method with Loeffler's mordant is as follows:

1. Air dry smears.
2. Physiological salt solution 10 minutes; dry.
3. Methyl alcohol; dry.
4. Steam with Loeffler's mordant; rinse well.
5. Antiformin 0.3% for ½ minute or 0.2% for 1 minute; rinse.
6. Stain with either carbol fuchsin 3 to 5 minutes, carbol methyl violet (6B) 15 minutes, saturated aqueous fuchsin or methylene blue solution 10 to 20 minutes. (Source of stains not indicated).

The method with the chromic acid mordant is:

1. Dry and flame the smears.
2. 5% chromic acid 10 to 30 minutes; rinse.
3. 0.3% antiformin about 1 minute; rinse.
4. Carbol fuchsin or carbol methyl violet (6B) 5 to 10 minutes; rinse.

Antiformin enables staining with more dyes than possible with the original Paschen's method. The Paschen bodies seem larger, clearer and sometimes are surrounded by light areas. Various decolorizing agents and many counterstains were tested, but none seemed quite satisfactory. Tartaric acid was preferred for destaining. Dependable differentiation between the bodies and tissue granules was not secured; but when numerous the former are easily recognized.

WEISS, EMIL. Method for staining of polar bodies. J. Lab. & Clin. Med., **15**, 170–171. 1929.

A method proposed for staining the polar bodies of bacteria represents a modification of the Neisser method and has the following advantages over the latter: it increases the size of the polar bodies and stains them more deeply; it shows up the bacterial cells more plainly. The procedure is as follows:

The smears are fixed over a flame as usual. The slides are covered 5 minutes with the following solution (methylene blue Grübler 5 g, 95% alcohol 10 cc., glacial acetic acid 5 cc., distilled water q.s. 100 cc.). The stain is then removed with running water, and the slides are covered for 1 to 2 minutes with 1% safranin or fuchsin 1:20 (source of these two dyes not mentioned). The slides are washed with water and dried. The metachromatic bodies appear deep blue, the bacterial cells distinctly red.

WOODS, M. W. Preserving certain algae in natural colors. Science, **70**, 637. 1929.

1. Soak 2 days in tap water (to remove some lime).
2. Remove air by soaking in cooled boiled water or in an aspirator.
3. Fix 4 hours in a solution containing 4% acetic acid and 0.5% C. P. copper acetate (or sulfate); agitate occasionally.
4. Pour off the solution and add to it just enough ammonia to give it a purple tint. Pour the solution back for an hour or until the material shows a blue green color.
5. Wash in tap water for 10 minutes. Soak in 50 volumes of distilled water for 5 minutes. The thallus should be bluish green and the antheridia orange, the oogonia brown and green. Transfer to 5% glycerin. If the latter causes plasmolysis, wash in 5% glycerin instead of the tap water.
6. Place material on pieces of smooth glass on slides in lots of size desired in the permanent mounts. Apply cover glasses and start evaporation at once. Use sunlight if available. Add 5%, 10% or thicker glycerin when necessary. The whole procedure should not take more than 10 to 12 hours if the colors are to be preserved.
7. When well concentrated remove the cover glasses and mount in Kaiser's gelatin. Let harden at least a week before sealing with balsam.

Plants other than algae may also be successfully preserved by this method.

ZIRKLE, C. The use of n-butyl alcohol in dehydrating woody tissue for paraffin embedding. Science, **71**, 103–104. 1930.

Xylol which hardens tissues and tends to shrink them may be omitted in the paraffin technic if butyl alcohol is used. The latter is soluble in paraffin, but very little in water. Equal parts of ethyl and butyl alcohol are completely mixable with water. The following schedule for dehydration (modification of Larbaud's) is proposed:

Water	95–89–82–70–50–30
Ethyl alcohol	5–11–18–30–40–50
Butyl alcohol	0– 0– 0– 0–10–20,

one hour in each solution and 24 hours in the one containing 70% alcohol. The alcohol not only dehydrates but also completes fixation by reducing the chromate. Dehydration is completed by the following stages:

Water	15– 5– 0– 0– 0
Ethyl alcohol	50–40–25– 0– 0
Butyl alcohol	35–55–75–100–100,

one hour in each and keep in the pure butyl alcohol until all the water has been extracted. Transfer material to vial two-thirds full of solid paraffin, cover with butyl alcohol and put in oven. Change paraffin twice, judging time by the size of the tissue. Butyl alcohol is less mixable with paraffin than is xylol and has decided advantages over the latter. Being lighter than melted paraffin it floats on the surface and thus hastens infiltration. It does not render paraffin crumbly, nor harden tissues. Wood that can be sectioned green can be sectioned by that method. *Smooth sections of cambium and phloem are obtained near the hard xylem.* The method is much preferred to celloidin for its greater rapidity and especially for the good cytologic fixation secured. It also can be used for other plant organs, such as buds, leaves, and root-tips.